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**Abstract:** Aims Extracts from pine tree bark containing a variety of flavonoids have been used in traditional medicine. Pycnogenol is a proprietary bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*) that exerts antioxidative, anti-inflammatory, and anti-platelet effects. However, the effects of Pycnogenol on endothelial dysfunction, a precursor of atherosclerosis and cardiovascular events, remain still elusive. Methods and results Twenty-three patients with coronary artery disease (CAD) completed this randomized, double-blind, placebo-controlled cross-over study. Patients received Pycnogenol (200 mg/day) for 8 weeks followed by placebo or vice versa on top of standard cardiovascular therapy. Between the two treatment periods, a 2-week washout period was scheduled. At baseline and after each treatment period, endothelial function, non-invasively assessed by flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound, biomarkers of oxidative stress and inflammation, platelet adhesion, and 24 h blood pressure monitoring were evaluated. In CAD patients, Pycnogenol treatment was associated with an improvement of FMD from  $5.3 \pm 2.6$  to  $7.0 \pm 3.1$  ( $P < 0.0001$ ), while no change was observed with placebo ( $5.4 \pm 2.4$  to  $4.7 \pm 2.0$ ;  $P = 0.051$ ). This difference between study groups was significant [estimated treatment effect 2.75; 95% confidence interval (CI): 1.75, 3.75,  $P < 0.0001$ ]. 15-F(2t)-Isoprostane, an index of oxidative stress, significantly decreased from  $0.71 \pm 0.09$  to  $0.66 \pm 0.13$  after Pycnogenol treatment, while no change was observed in the placebo group (mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11),  $P = 0.012$ ). Inflammation markers, platelet adhesion, and blood pressure did not change after treatment with Pycnogenol or placebo. Conclusion This study provides the first evidence that the antioxidant Pycnogenol improves endothelial function in patients with CAD by reducing oxidative stress. Clinical Trial Registration: ClinicalTrials.gov identifier: NCT00641758.

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# Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study

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## Aims

Extracts from pine tree bark containing a variety of flavonoids have been used in traditional medicine. Pycnogenol is a proprietary bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*) that exerts antioxidative, anti-inflammatory, and anti-platelet effects. However, the effects of Pycnogenol on endothelial dysfunction, a precursor of atherosclerosis and cardiovascular events, remain still elusive.

## Methods and results

Twenty-three patients with coronary artery disease (CAD) completed this randomized, double-blind, placebo-controlled cross-over study. Patients received Pycnogenol (200 mg/day) for 8 weeks followed by placebo or vice versa on top of standard cardiovascular therapy. Between the two treatment periods, a 2-week washout period was scheduled. At baseline and after each treatment period, endothelial function, non-invasively assessed by flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound, biomarkers of oxidative stress and inflammation, platelet adhesion, and 24 h blood pressure monitoring were evaluated. In CAD patients, Pycnogenol treatment was associated with an improvement of FMD from  $5.3 \pm 2.6$  to  $7.0 \pm 3.1$  ( $P < 0.0001$ ), while no change was observed with placebo ( $5.4 \pm 2.4$  to  $4.7 \pm 2.0$ ;  $P = 0.051$ ). This difference between study groups was significant [estimated treatment effect 2.75; 95% confidence interval (CI): 1.75, 3.75,  $P < 0.0001$ ]. 15-F<sub>2t</sub>-Isoprostane, an index of oxidative stress, significantly decreased from  $0.71 \pm 0.09$  to  $0.66 \pm 0.13$  after Pycnogenol treatment, while no change was observed in the placebo group (mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11),  $P = 0.012$ ). Inflammation markers, platelet adhesion, and blood pressure did not change after treatment with Pycnogenol or placebo.

## Conclusion

This study provides the first evidence that the antioxidant Pycnogenol improves endothelial function in patients with CAD by reducing oxidative stress.

Clinical Trial Registration: ClinicalTrials.gov identifier: NCT00641758

## Keywords

Pycnogenol • Coronary artery disease • Clinical trial • Endothelial function • Oxidative stress

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## Introduction

Extracts from pine tree bark have been used in traditional medicine. Pycnogenol is a bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*). The extract is prepared using a standardized procedure that includes an extraction of fresh pine bark with ethanol and water according to the requirements published by the United States Pharmacopoeia (USP).<sup>1</sup> The resulting product is a mixture of flavonoids as monomers, i.e. catechin and taxifolin, and condensed polymers (85%), as well as dodecameric flavonols, designated as procyanidins, differing in structure and chain length. Procyanidins are biopolymers composed of catechin and epicatechin, widely distributed in the plant kingdom. Many edible fruits contain procyanidins, e.g. apples, berries, peanuts, and grapes. Additionally, the extract contains phenolic acids as gallic, caffeic, and ferulic acid as minor constituents. Pycnogenol also contains glycosylation products, i.e. sugar derivatives of phenolcarboxylic acids and taxifolin.<sup>2</sup> These molecules have antioxidant properties and may act as modulators of metabolic enzymes and other cellular functions.<sup>2</sup>

In a pharmacokinetic study, healthy volunteers ingested a single dose of 300 mg and repeated doses of 200 mg Pycnogenol.<sup>3</sup> In blood samples, catechin, taxifolin, ferulic acid, caffeic acid, and the metabolite 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone (M1) were detected in concentrations up to 100 ng/mL. While the first four compounds are genuinely present in the maritime pine bark extract, the metabolite M1 is formed *in vivo* from catechin polymers by gut microbiota.<sup>4,5</sup> This metabolite had been previously also found in urine samples after Pycnogenol intake.<sup>6</sup> Additionally to these known constituents and metabolites, 10 yet unknown compounds were detected in human plasma samples.<sup>7</sup>

The effects of the identified compounds have been investigated *in vitro* with the metabolite M1 revealing the highest activity. On a microgram per millilitre basis, M1 was more potent than the original maritime pine bark extract in inhibiting the activity of matrix metalloproteinase (MMP)-1, -2 and -9.<sup>3</sup> M1 was also about twice as potent as hydrocortisone in inhibiting the release of MMP-9 from freshly isolated human monocytes after stimulation with bacterial lipopolysaccharide. Thus, it appears very likely that M1 plays a major role in mediating the biological effects of Pycnogenol.

However, it cannot be excluded that other yet unknown components or metabolites of Pycnogenol also contribute to its biological activity. Plasma samples of volunteers who ingested Pycnogenol contained bioactive compounds which in addition to the inhibition of MMP-9 release statistically significantly inhibited nuclear factor kappa B (NF- $\kappa$ B) activation<sup>8</sup> as well as cyclooxygenase 1 and 2 activity.<sup>9</sup>

Pycnogenol has been shown to exert anti-inflammatory effects and to inhibit platelet aggregation, both risk factors for cardiovascular disease.<sup>2</sup> *In vitro*, Pycnogenol prolonged the ascorbate radical lifetime to the greatest extent by regenerating ascorbic acid.<sup>10</sup> Similarly, Pycnogenol inhibits LDL peroxidation, lipid peroxidation in phospholipid liposomes,<sup>2</sup> and lipid peroxidation caused by *t*-butylhydroperoxide. Furthermore, Pycnogenol has antioxidant activity.<sup>10</sup> Finally, in isolated blood vessels, Pycnogenol inhibits vasoconstriction caused by adrenalin via the production of nitric oxide.<sup>11</sup> The present study was designed to evaluate the effect

of Pycnogenol on endothelial function in patients with coronary artery disease (CAD).

## Methods

### Study population

Patients with CAD, documented by coronary angiography, nuclear imaging, or a positive stress test, on stable cardiovascular medication for at least 1 month and  $\geq 18$  years of age, who gave written informed consent, were recruited at the Clinic of Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland.

Exclusion criteria were acute myocardial infarction, unstable angina, stroke, or coronary intervention/revascularization procedure within 3 months prior to study entry; uncontrolled symptomatic congestive heart failure (New York Heart Association functional class  $> \text{II}$ ) in the last 4 weeks prior to study entry; smoking, alcohol, or illicit substance abuse; uncontrolled blood pressure despite adequate therapy ( $\geq 160/100$  mmHg); symptomatic hypotension; ventricular tachyarrhythmias; any cardiomyopathy, untreated thyroid dysfunction, or adrenal insufficiency; renal failure (creatinine clearance using the MDRD formula<sup>12</sup>  $< 50$  mL/min); liver disease; chronic use of long-acting nitrates; oral or intravenous steroid therapy; insulin-dependent diabetes mellitus; anaemia (Hb  $< 10$  g/dL); known hypersensitivity to Pycnogenol; systemic inflammatory diseases (e.g. rheumatoid arthritis, Crohn's disease); known human immunodeficiency virus infection or active virus—hepatitis; pregnancy or breast-feeding, women with childbearing potential without adequate contraception; malignancy (unless healed or in remission  $> 5$  years); recipient of any major organ transplant (e.g. lung, liver, heart) or renal replacement therapy and the participation in another study within the last month.

### Study design and protocol

In this single-centre, prospective, randomized, double-blind, placebo-controlled study in a cross-over fashion, we analysed the impact of Pycnogenol on endothelium-dependent and -independent dilatation in patients with stable CAD receiving optimal standard therapy. Pre-specified secondary endpoints were change in high-sensitive C-reactive protein and CD40 Ligand; change in total antioxidative capacity, oxidized LDL (oxLDL), and 8-isoprostanes; change in ambulatory blood pressure (ABP); and change in shear stress-dependent platelet function. The protocol was approved by the Institutional Review Board (Ethics Committee of the Canton Zurich) and the Swiss Agency for Therapeutic Products (Swissmedic, Bern, Switzerland). The study was registered at ClinicalTrials.gov (Identifier: NCT00641758).

After screening and recruiting, the patients were randomly assigned into two groups. For randomization, an unpredictable allocation sequence was provided by external institutions (InterCorNet, Zurich, Switzerland), which were responsible for the blinding and labelling of the drugs. The study drug and placebo were prepared in identical capsules to ensure uniform appearance of both formulations by the manufacturer Horphag Research (UK) Ltd, London, UK. All investigators including the ultrasonographers were unaware of the allocation procedure at any time. The patients were randomized to receive either Pycnogenol 200 mg/day or matching placebo in the first part or vice versa in the second part with a washout period of 2 weeks in-between.

At each visit (baseline and after 8, 10, and 18 weeks), endothelial function and ABP were measured, blood samples were drawn, the clinical status was assessed, and adverse events were recorded. At each visit, a safety analysis was performed including assessment of electrolytes, of liver and kidney function, as well as a white and red blood cell count. Pregnancy testing in women with childbearing potential

was performed only at the first visit. The patients were advised not to take their usual drugs in the morning of the examination day (all examinations and measurements were performed in the morning); moreover, the medical therapy was unchanged throughout the study. Blood samples and the evaluation of flow-mediated dilatation (FMD) were performed before the patients took their medications. The regular medications and the study drug were taken thereafter and before the 24 h ABP measurement was placed.

## Endothelium-dependent and -independent vasodilatation

Flow-mediated dilatation was performed according to the current guidelines<sup>13,14</sup> and as described previously.<sup>15</sup> In brief, a B-mode high-resolution ultrasound scan of the left brachial artery was obtained by highly trained and experienced sonographers in a longitudinal section between 2 and 10 cm above the elbow, using a high-resolution 10 MHz linear array transducer and a high-resolution ultrasound system Siemens X300 (Siemens Switzerland AG, Zurich, Switzerland). The analogue video signal was acquired with a video processing system that computed the artery diameter in real-time (FMD Studio,<sup>16</sup> a system for real-time measurement, Institute of Clinical Physiology, Pisa, Italy). The high reproducibility of the method has been demonstrated recently.<sup>17,18</sup> The baseline vessel size was considered as the mean of the measures obtained during the first minute. Flow-mediated dilatation was calculated as the maximal per cent increase in diameter above the baseline. Endothelium-independent dilatation was measured after sublingual glycerol trinitrate (GTN; 0.4 mg, Nitrolingual Spray, Pohl-Boskamp, Hohenlockstedt, Germany) application by recording arterial diameter continuously for at least 6 min. The response to GTN is calculated as the maximum per cent increase in vessel size above the baseline. The reproducibility of our laboratory was published previously.<sup>15</sup>

## Special laboratory analysis

### Oxidative stress markers

8-Epi-PGF2 $\alpha$  (15-F<sub>2t</sub>-isoprostane) was measured in the plasma with an 8-Isoprostane Enzyme Immunoassay [8-isoprostane Express EIA Kit, Cayman Chemicals, Ann Arbor, MI, USA; intra-assay coefficient of variation (CV) 7.2%, inter-assay CV 15.5%].

### High-sensitivity C-reactive protein

A high-sensitive immunoluminometric assay (Imulite 2002, DCP, Los Angeles, CA, USA) was used to measure high-sensitivity C-reactive protein levels as described by Wood *et al.*<sup>19</sup>

### Soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, soluble CD40 Ligand, and oxidized low-density lipoprotein

EDTA plasma was drawn from each patient and immediately frozen at  $-70^{\circ}\text{C}$ . Plasma samples were thawed only once for aliquotation before they were subjected to analyses. Quantitative detection of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble CD40 Ligand (sCD40L) (all Bender Med Systems, Vienna, Austria), oxLDL (Immunodiagnostik, Bernsheim, Germany), and of Lipoprotein-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) (Uscn Life Sciences Inc., Wuhan, China) was performed using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Briefly, pre-coated microwell plates absorbed with specific capture antibodies directed against the respective molecule were washed thoroughly with the washing buffers provided. Plasma samples were diluted where

indicated and applied in duplicates before a 1–2 h incubation at room temperature. Detection of the target molecules was performed using a horseradish peroxidase-conjugated detection antibody specific for the respective molecule and visualized during a colour-giving reaction using the provided TMB substrate solution. Absorption was measured at 450 nm and values were normalized to the respective internal standards supplied by the manufacturer.

### Total antioxidant capacity

Total antioxidant capacity of plasma was measured using the total antioxidant capacity kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, plasma was allowed to reduce Cu<sup>2+</sup> for 1.5 h at room temperature. Reduced Cu<sup>+</sup> was chelated with a colorimetric probe and absorbance was measured at 570 nm. Results were expressed as trolox equivalent according to a trolox standard curve.

### Endothelin-1

The endothelin-1 (ET-1) sandwich ELISA system from Assay Designs (Ann Arbor, MI, USA) was used according to the manufacturer's instructions to measure ET-1 protein concentrations in EDTA plasma samples. Briefly, 500  $\mu\text{L}$  of each plasma sample was extracted with 650  $\mu\text{L}$  extraction solvent [acetone:1 N HCl:water (40:1:5)] and dried under nitrogen streaming. Reconstituted in 120  $\mu\text{L}$  assay buffer, samples were employed for testing. The sensitivity of the assay was determined to be 0.41 pg/mL, the intra-assay CVs were 6.7% at 35.9 pg/mL, 8.9% at 2.3 pg/mL, and 8.8% at 1.1 pg/mL; cross-reactivity with human Big ET-1, ET-2, and ET-3 was low (human Big ET-1 < 0.1%, ET-2 = 21%, and ET-3 = 3.6%).

### Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) concentration was measured from plasma samples by a competitive ELISA system including an acylation step as pre-treatment (DLD Diagnostika GmbH, Hamburg, Germany), with a standard range from 0.1 to 5.0  $\mu\text{mol/L}$ . Twenty microlitres of each plasma sample were employed for testing. The detection limit of the system was 0.05  $\mu\text{mol/L}$ . As described previously,<sup>20</sup> the intra-assay CVs were 5.7% at 0.66  $\mu\text{mol/L}$  and 6.4% at 1.01  $\mu\text{mol/L}$ ; the inter-assay CV ranged from 8.3 to 10.3%. Cross-reactivity with arginine and other methylarginines was low [arginine < 0.02%; NG-monomethyl-L-arginine 1.0%; symmetric dimethylarginine (SDMA) 1.2%]. The correlation coefficients with liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry were high (0.991; 0.984), with a good linearity between 0.1 and 5.0 mol/L.

### Symmetric dimethylarginine

Symmetric dimethylarginine was measured from serum samples by a competitive ELISA system including an acylation step as pre-treatment (DLD Diagnostika GmbH) with a standard range from 0.08 to 3.0  $\mu\text{mol/L}$ . Twenty microlitres of each plasma sample were employed for testing. The detection limit of the system was 0.05  $\mu\text{mol/L}$ . The intra-assay CVs were 5.7% at 0.52  $\mu\text{mol/L}$ , 6.1% at 0.75  $\mu\text{mol/L}$ , and 4.7% at 1.72  $\mu\text{mol/L}$ ; cross-reactivity with arginine and other methylarginines was low (arginine < 0.01%; NG-monomethyl-L-arginine 0.70%; ADMA 0.44%).

## Ambulatory blood pressure measurement

Ambulatory blood pressure measurements were obtained over 24 h using the 'Tracker NIBP 2' (Delmar, Del Mar Reynolds Medical, Hertford, UK) before and after the active treatment phase according to recent guidelines.<sup>21</sup> Patients were asked to keep their arm calm while the cuff was inflating and to avoid excessive physical exertion



during monitoring. The monitors were programmed to take readings every 15 min during daytime and every 30 min during night-time.

## Shear stress-dependent platelet function

Shear stress-dependent platelet function was assessed with a cone and platelet analyzer as described previously.<sup>15,22</sup>

## Statistical analysis

The primary endpoint was the change in FMD after 8 weeks of treatment with Pycnogenol compared with placebo. Based on previous studies,<sup>23</sup> the estimated sample size for this study is 25 (to show a difference of 1.3% in FMD with a standard deviation of 1.5%, giving 5% two-sided significance level and 85% power when using a *t*-test). Analysis was performed using the Wilcoxon and Mann–Whitney *U*-tests (to account for possible non-normality of the endpoints), using methods discussed by Senn.<sup>24</sup> That is, we considered two distinct groups of patients: Group 1 who received Pycnogenol followed by placebo and Group 2 who received placebo followed by Pycnogenol. Within each group, data are summarized by examining within patients changes between Periods 1 and 2 (for Group 1, this is the change from baseline while on placebo minus the change in baseline while on Pycnogenol; for Group 2, the change from baseline while on Pycnogenol minus the change in baseline while on placebo). These unpaired change scores are then analysed using an unpaired Wilcoxon and the Mann–Whitney *U*-test, as proposed by Hill and Armitage,<sup>25</sup> and later discussed by Senn.<sup>24</sup> Statistical tests were performed two-sided. The effect of Pycnogenol is estimated as the average of the two group-specific mean change scores. The period effect is estimated using the difference in the two group-specific mean change scores (divided by 2). The carry-over effect was excluded using an unpaired Wilcoxon test of within-patient change from baseline including only the first period of treatment. A Bonferroni–Holm<sup>26</sup> correction was made if necessary. For the ‘responder analysis’, patients were divided evenly into groups based on their FMD to Pycnogenol and placebo. Those with better responses were considered ‘responders’ and the others, ‘non-responders’. The analyses for C-reactive protein and 15-F<sub>2t</sub>-isoprostane were then performed again separately for the two groups. Results are presented as mean  $\pm$  SD or SEM as described. Analysis of the primary endpoint was performed in the R programming language (R Development Core Team, 2009). The statistical software package SPSS 17 (SPSS Inc., Chicago, IL, USA) was used to evaluate difference in the clinical characteristics. Statistical significance was accepted at  $P < 0.05$ .

## Results

### Study population

A total of 28 patients were enrolled, 5 patients discontinued the study prematurely, 3 patients withdrew consent and 2 patients were excluded for adverse events: one patient in the Pycnogenol group was hospitalized for acute decompensated heart failure; one patient in the placebo group was excluded after development of an itching rash. Therefore, 23 patients (19 males and 4 females) with a mean age of  $63.1 \pm 7.1$  years (range: 49–73 years) and a body mass index of  $27.3 \pm 3.3$  kg/m<sup>2</sup> completed the study protocol and were included into the analysis. The mean left ventricular ejection fraction was  $62 \pm 10\%$ . With the included 23 patients, the statistical power of 80% is sufficient to detect differences of 1.3% in

FMD between the two treatments. All patients had stable CAD, 11 patients with one-vessel disease, 1 patient with two-vessel disease, and 11 patients with three-vessel disease.

### Effect of Pycnogenol on endothelium-dependent and -independent vasodilatation

After 8-week treatment with Pycnogenol, FMD increased from  $5.3 \pm 2.6$  to  $7.0 \pm 3.1\%$ , while it remained unchanged in the placebo group ( $5.4 \pm 2.4$  to  $4.7 \pm 2.0\%$ ; Table 1 and Figure 1). The estimated effect of Pycnogenol on FMD was an increase of 2.75 compared with placebo [mean difference 2.75 with an associated 95% confidence interval (CI: 1.75, 3.74),  $P < 0.0001$ ], a statistically significant treatment effect. The period effect for this endpoint was estimated to be  $-0.32$  [95% CI ( $-1.31$ ,  $0.68$ ),  $P = 0.51$ ] and, therefore, not statistically significant. The endothelium-independent dilatation induced by GTN remained unchanged before and after treatment with Pycnogenol (Table 1).

### Effect of Pycnogenol on ambulatory blood pressure

Systolic and diastolic ABP remained unchanged before and after treatment with Pycnogenol (Table 2). The estimated effect of Pycnogenol on systolic blood pressure was an increase of 1.17 mmHg compared with placebo [mean difference 1.17 mmHg with associated 95% CI ( $-4.71$ ,  $7.04$ ),  $P = 0.68$ ], a statistically non-significant treatment effect. The estimated effect of Pycnogenol on diastolic blood pressure was an increase of 0.06 mmHg compared with placebo [mean difference 0.06 mmHg with associated 95% CI ( $-3.26$ ,  $3.37$ ),  $P = 0.97$ ], a statistically non-significant treatment effect.

### Effect of Pycnogenol on oxidative stress

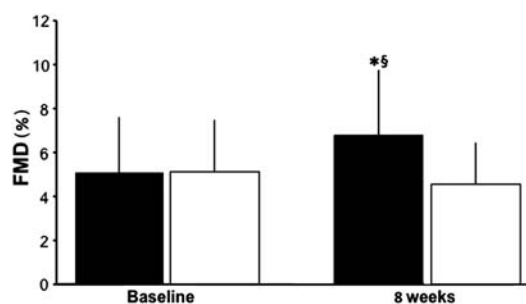
15-F<sub>2t</sub>-Isoprostane was reduced significantly after 8-week treatment with Pycnogenol compared with baseline and placebo (Table 2 and Figure 2). The estimated effect of Pycnogenol on 15-F<sub>2t</sub>-Isoprostane was a decrease of 0.06 pg/mL compared with placebo [mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11),  $P = 0.012$ ], a statistically significant treatment effect. This effect was even more pronounced, when the patients, ‘responders’ and ‘non-responders’, were analysed. The estimated effect of Pycnogenol on 15-F<sub>2t</sub>-Isoprostane (responders) was a decrease of 0.1 compared with placebo [mean difference 0.1 with associated 95% CI (0.00, 0.20),  $P = 0.045$ ], a statistically significant treatment effect, while no change was observed in the ‘non-responders’. The estimated effect of Pycnogenol on 15-F<sub>2t</sub>-isoprostane (non-responders) was an increase of 0.04 compared with placebo [mean difference 0.04 with associated 95% CI ( $-0.02$ ,  $0.10$ ),  $P = 0.15$ ], which was not statistically significant.

### Effect of Pycnogenol on markers of inflammation

The estimated effect of Pycnogenol on C-reactive protein was an increase of 2.18 mg/L compared with placebo [mean difference 2.18 mg/L with associated 95% CI ( $-3.35$ ,  $7.71$ ),  $P = 0.42$ ], a statistically non-significant treatment effect. Moreover, all of the

**Table 1** Clinical measures before and after 8-week treatment with Pycnogenol or placebo and concomitant medication

	Baseline for treatment period	Pycnogenol	Baseline for control period	Placebo
Clinical parameters				
FMD (%)	5.3 ± 2.6	7.0 ± 3.1* <sup>#</sup>	5.4 ± 2.4	4.7 ± 2.0
GTN (%)	18.4 ± 5.4	19.4 ± 4.8	19.5 ± 5.4	19.7 ± 5.7
24 h SBP (mmHg)	125.8 ± 8.7	125.5 ± 8.1	124.8 ± 9.7	125.7 ± 8.1
24 h DBP (mmHg)	75.0 ± 7.0	74.3 ± 7.3	73.9 ± 7.5	74.4 ± 6.6
Office SBP (mmHg)	129.9 ± 12.5	131.8 ± 14.5	130.1 ± 12.1	129.8 ± 11.7
Office DBP (mmHg)	77.4 ± 6.8	78.9 ± 7.7	78.9 ± 7.5	79.7 ± 8.0
Office HR (b.p.m.)	58.3 ± 8.3	59.4 ± 9.0	60.3 ± 12.3	57.6 ± 9.9
Concomitant medication				
Aspirin	23/23 (100%)			
Statin	20/23 (87%)			
ACE-inhibitor/ARB	18/23 (78%)			
β-Blocker	17/23 (74%)			
Diuretics	8/23 (35%)			
Calcium-antagonist	4/23 (17%)			
Clopidogrel	4/23 (17%)			
Ezetimibe	4/23 (17%)			
Oral anti-diabetics	4/23 (17%)			
Marcoumar	1/23 (4%)			
α-Antagonist	1/23 (4%)			

\**P* < 0.05 vs. baseline.<sup>#</sup>*P* < 0.05 vs. placebo.**Figure 1** Flow-mediated dilatation (FMD, %) of the brachial artery in patients receiving Pycnogenol (black bar) or placebo (white bar). \**P* < 0.05 vs. baseline and <sup>§</sup>*P* < 0.05 vs. placebo.

following parameters remained unchanged before and after treatment with Pycnogenol or placebo (Table 2): total antioxidant capacity [mean difference  $-0.19 \mu\text{M}$  with associated 95% CI  $(-2.20, 1.83)$ ,  $P = 0.85$ ], sVCAM [mean difference  $-20.44 \text{ ng/mL}$  with associated 95% CI  $(-128.88, 88.00)$ ,  $P = 0.7$ ], sICAM [mean difference  $-67.38 \text{ ng/mL}$  with associated 95% CI  $(-141.83, 7.07)$ ,  $P = 0.073$ ], sCD40L [mean difference  $0.02 \text{ ng/mL}$  with associated 95% CI  $(-0.21, 0.25)$ ,  $P = 0.85$ ], and oxLDL [mean difference  $-2.56 \text{ ng/mL}$  with associated 95% CI  $(-18.59, 13.47)$ ,  $P = 0.74$ ], and Lp-PLA<sub>2</sub> [mean difference  $74.83 \text{ ng/mL}$  with associated 95% CI  $(-46.10, 195.76)$ ,  $P = 0.21$ ].

### Effect of Pycnogenol on symmetric dimethylarginine, asymmetric dimethylarginine, and endothelin-1

The estimated effect of Pycnogenol on SDMA was a decrease of  $0.06 \mu\text{M}$  compared with placebo [mean difference  $-0.06 \mu\text{M}$  with associated 95% CI  $(-0.16, 0.04)$ ,  $P = 0.23$ ], a statistically non-significant treatment effect. The estimated effect of Pycnogenol on ADMA was a decrease of  $0.05 \mu\text{M}$  compared with placebo [mean difference  $-0.05$  with associated 95% CI  $(-0.12, 0.03)$ ,  $P = 0.19$ ]. Though 57% of the patients receiving Pycnogenol and only 30% of the patients taking placebo revealed lower ADMA concentrations after the respective treatment period, the effect was not statistically significantly different (Table 2).

The mean ET-1 concentrations of the participants were throughout at the lower limit of the reported physiological range between  $1.10$ – $2.70 \text{ ng/L}$ <sup>27</sup> and no statistically significant treatment effect was observed (Table 2).

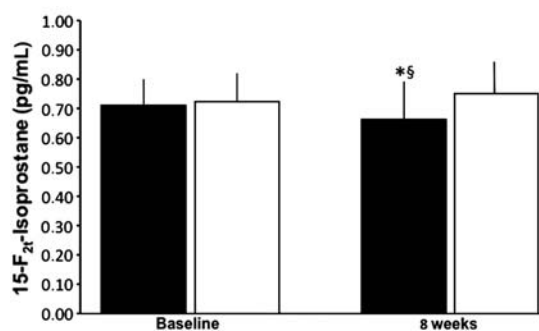
### Effect of Pycnogenol on shear stress-dependent platelet function

Shear stress-dependent platelet function did not change after treatment with Pycnogenol (Table 2). The estimated effect of Pycnogenol on area fraction was a decrease of  $0.22$  compared with placebo [mean difference  $-0.22$  with associated 95% CI  $(-1.85, 1.42)$ ,  $P = 0.78$ ], a statistically non-significant treatment effect.

**Table 2** Laboratory values before and after treatment with Pycnogenol or placebo

	Baseline before treatment period	Pycnogenol	Baseline before control period	Placebo
Hb (g/L)	14.5 ± 1.2	14.5 ± 1.2	14.6 ± 1.2	14.7 ± 1.2
Hk (%)	41.4 ± 3.2	41.3 ± 3.8	41.1 ± 3.4	41.6 ± 3.3
Sodium (mmol/L)	141.1 ± 2.3	141.6 ± 2.2	141.4 ± 2.1	141.9 ± 2.1
Potassium (mmol/L)	3.9 ± 0.3	3.8 ± 0.4	3.9 ± 0.4	3.8 ± 0.4
Creatinine (μmol/L)	91.7 ± 13.9	90.6 ± 25	91.6 ± 16.7	93.4 ± 14.1
Glucose (mmol/L)	5.7 ± 1.4	5.6 ± 1.3	5.7 ± 1.1	5.7 ± 1.3
TC (mmol/L)	4.6 ± 0.6	4.6 ± 0.7	4.7 ± 0.7	4.8 ± 0.8
HDL-C (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
LDL-C (mmol/L)	2.5 ± 0.5	2.5 ± 0.6	2.6 ± 0.6	2.7 ± 0.7
TG (mmol/L)	1.4 ± 0.7	1.5 ± 0.7	1.5 ± 0.9	1.5 ± 0.9
ALT (U/L)	27.7 ± 4.9	30.0 ± 7.0	29.4 ± 6.3	29.2 ± 5.4
AST (U/L)	29.5 ± 8.2	31.6 ± 9.3	92.6 ± 9.7	32.9 ± 9.9
High-sensitivity C-reactive protein (mg/L)	1.52 ± 1.37	1.52 ± 1.33	1.63 ± 1.71	1.91 ± 3.4
Lp-PLA <sub>2</sub> (ng/mL)	315.89 ± 23.5	321 ± 27.7	279.4 ± 20.4	368.6 ± 24.8
15-F <sub>2t</sub> -Isoprostane (pg/mL)	0.71 ± 0.09	0.66 ± 0.13* <sup>#</sup>	0.72 ± 0.09	0.75 ± 0.10
ADMA (μM)	0.47 ± 0.11	0.49 ± 0.11	0.45 ± 0.12	0.50 ± 0.12
SDMA (μM)	0.47 ± 0.13	0.47 ± 0.13	0.45 ± 0.12	0.51 ± 0.13
Endothelin-1 (ng/L)	1.20 ± 0.36	1.27 ± 0.30	1.23 ± 0.27	1.21 ± 0.30
Platelet adhesion (%)	3.84 ± 2.0	3.49 ± 1.45	3.2 ± 1.6	3.2 ± 2.0
Total antioxidant capacity (μM)	10.12 ± 1.65	10.1 ± 1.28	9.65 ± 2.72	9.78 ± 2.70
sVCAM (ng/mL)	869 ± 232	791 ± 226	824 ± 265	783 ± 244
sICAM (ng/mL)	284 ± 90	232 ± 77	277 ± 91	274 ± 130
sCD40L (ng/mL)	0.54 ± 1.11	0.50 ± 1.0	0.54 ± 1.10	0.46 ± 1.01
OxLDL (ng/mL)	77.9 ± 119.0	78.9 ± 123.5	85.8 ± 155.4	88.5 ± 154.0

\*P &lt; 0.05 vs. baseline.

<sup>#</sup>P < 0.05 vs. placebo.**Figure 2** 15-F<sub>2t</sub>-Isoprostane before and after Pycnogenol (black bar) or placebo (white bar); \*P < 0.05 vs. baseline and <sup>§</sup>P < 0.05 vs. placebo.

## Discussion

This study demonstrates an improvement of endothelial function after 8-week treatment with the flavonoid extract Pycnogenol at a dose of 200 mg q.d. when compared with placebo in patients with stable CAD.

The balance between endothelial function and dysfunction plays an important role in the initiation and progression of atherosclerosis and in the transition from a stable to an unstable disease state.<sup>28</sup> As such, the evaluation of endothelial function has emerged as an important endpoint in cardiovascular research. A recent meta-analysis suggests that an impairment of brachial FMD is strongly associated with the occurrence of future cardiovascular events.<sup>29</sup> Therefore, preservation or recovery of endothelial function is an important therapeutic aim in the prevention of arteriosclerosis and its clinical complications such as myocardial infarction and stroke. The improvement of endothelial function in our study was associated with a reduction in 8-isoprostanes, a marker for oxidative stress, but not with components of the L-arginine metabolism or markers of inflammation, indicating that antioxidant properties of the compound appear to be involved.

An improvement of endothelial function has been demonstrated by Nishioka et al.<sup>30</sup> in a randomized, double-blind, placebo-controlled study, including 16 healthy young men who were randomized to receive either Pycnogenol (180 mg/day) or placebo for 2 weeks. The investigators measured forearm blood flow (FBF) using strain-gauge plethysmography. They found an augmented FBF response to acetylcholine (ACh) in the Pycnogenol group, while no augmentation was seen in the placebo group. However,



sodium-nitroprusside response was similar in both treatment groups and the administration of NG-monomethyl-L-arginine, a known nitric oxide synthase (NOS) inhibitor, abolished the Pycnogenol-induced augmentation to FBF response to ACh, suggesting an endothelium-dependent vasodilatation by increased NO production.

Several lines of evidence suggest that oxidative stress may promote endothelial dysfunction through different mechanisms.<sup>31</sup> First, diverse reactive oxygen species (ROS) are produced at increased levels within the vessel wall and they individually or in combination interfere with the function of endothelial and vascular smooth muscle cells.<sup>32</sup> The family of ROS includes highly bioactive, short-living molecules that are derived from a reduction in molecular oxygen. Multiple enzyme systems use different substrates as sources of electrons to produce a variety of ROS, the most important of which are NO, superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and peroxynitrite ( $ONOO^-$ ). Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and uncoupled NOS among others, contribute to production as well as to degradation of ROS.<sup>33</sup>  $ONOO^-$  is an important mediator of lipid peroxidation and protein nitration, including oxidation of LDL, which has potent proatherogenic effects.<sup>32</sup> NO is a crucial mediator of endothelium-dependent vasodilatation and also plays a role in platelet aggregation and in maintaining the balance between smooth muscle cell growth and differentiation.<sup>34</sup> NO is rapidly inactivated by  $O_2^-$  to form  $ONOO^-$ , a known mechanism of endothelial dysfunction.

The short half-life of ROS makes them ideal signalling molecules, but it also confounds their measurement in complex biological systems, particularly in humans *in vivo*.<sup>32</sup> On the other hand, F<sub>2</sub>-isoprostanes are associated with oxidative stress and are reliable markers of *in vivo* lipid peroxidation.<sup>35</sup> Isoprostanes (iPs) are a family of prostaglandin (PG)-like compounds formed non-enzymatically through free radical catalysed attack on esterified arachidonate followed by enzymatic release from cellular or lipoprotein phospholipids.<sup>32</sup> The measurement of F<sub>2</sub>-isoprostanes (F<sub>2</sub>-iPs), containing F-type ring analogous to PGF<sub>2 $\alpha$</sub> , provides a reliable tool for identifying populations with enhanced rates of lipid peroxidation.<sup>32</sup> In addition to their potential usefulness as indexes of oxidative stress *in vivo*, 8-iso-PGF<sub>2 $\alpha$</sub>  is a vasoconstrictor and modulates platelet activation in response to other agonists.<sup>32</sup> Interestingly, in our study, 8-iso-PGF<sub>2 $\alpha$</sub>  plasma levels decreased significantly by  $6.43 \pm 16.3\%$  after therapy with Pycnogenol ( $P = 0.012$ ) when compared with placebo (increase of  $4.54 \pm 11.1\%$ ). Moreover, the pronounced improvement of endothelial function after 8 weeks of treatment with Pycnogenol in the 'responder' group was paralleled by a significant decrease in 15-F<sub>2t</sub>-isoprostane, while in patients in the 'non-responder' group, no significant change was detected. These findings are in line with a study performed by Ryan *et al.*,<sup>36</sup> who examined the effects of Pycnogenol on different cognitive and biochemical parameters in a healthy elderly population (60–85 years old). In this double-blind, placebo-controlled, matched-pair study, 101 participants received Pycnogenol (150 mg/day) for 3 months. The participants in the Pycnogenol group showed an improved working memory and decreased levels of F<sub>2</sub>-isoprostanes compared with the control group.

Indeed, Pycnogenol is able to scavenge both hydroxyl radicals and superoxide anions<sup>37</sup> and increase the activity of superoxide dismutase, glutathione peroxidase, and catalase.<sup>38</sup> These *in vitro* effects have been confirmed in healthy subjects, treated with Pycnogenol 150 mg q.d.<sup>39</sup> In that study oxygen radical absorption capacity increased by 40% during treatment and returned to baseline after washout. However, there was no significant change in plasma lipid peroxidation or in LDL-cholesterol oxidation.<sup>39</sup> These data are in line with our study, showing no changes in plasma lipids including oxLDL or in total antioxidative capacity.

Endothelial dysfunction has been linked to elevated blood levels of ADMA, which is an endogenous inhibitor of NO synthesis.<sup>40,41</sup> Even small changes of ADMA concentration alter vascular NO production. Symmetric dimethylarginine correlates with renal function and indicates an increased cardiovascular risk.<sup>42,43</sup> It has been shown to increase production of ROS and is associated with atrial fibrillation and endothelial and platelet activation. Although ADMA plasma levels tended to be lower after Pycnogenol treatment, neither the effect on ADMA nor SDMA concentrations reached statistical significance. Although the L-arginine/NO metabolism was not studied in particular, our finding strongly suggests that alterations in the L-arginine/NO metabolisms are unlikely involved in the endothelial protective effects of Pycnogenol.

Endothelin-1 is a potent vasoconstrictor modulating the release and action of NO in the vessel wall.<sup>44</sup> Furthermore, previous studies suggested<sup>45</sup> that Pycnogenol may lower the plasma levels of the peptide. However, in the current study, no statistically significant effect of the flavonoid extract could be observed. This might be due to the fact that all participants, although CAD patients, exhibited low ET-1 plasma levels at the lower limit of the physiological reference range. Thus, it cannot be excluded that Pycnogenol might affect vascular ET-1 tissue levels.

The anti-inflammatory properties of Pycnogenol have been demonstrated in a large variety of studies, both *in vitro* and *in vivo*.<sup>46</sup> Pycnogenol is able to inhibit tumour necrosis factor- $\alpha$ -induced NF- $\kappa$ B activation and adhesion molecule expression in human umbilical vein endothelial cells.<sup>47</sup> In two double-blind, randomized, placebo-controlled trials in patients with asthma, the investigators found improved asthma symptom scores as well as improved pulmonary function, which was paralleled by a reduction in plasma, as well as in urine leucotriene concentrations after Pycnogenol treatment.<sup>48,49</sup> While we could demonstrate a marked improvement in endothelial function in stable CAD patients treated with Pycnogenol for 8 weeks, which was paralleled by a decrease in 15-F<sub>2t</sub>-isoprostane, we could not demonstrate an improvement in inflammatory parameters (high-sensitivity C-reactive protein, sVCAM, sICAM, and sCD40L) or total antioxidative capacity. This lack of effect may be due to the sample size, which of course was calculated for the primary endpoint.

The effects of Pycnogenol on platelet function were investigated by Araghi-Niknam *et al.*<sup>50</sup> in 60 patients with stable CAD, using the PFA-100 test with adenosine diphosphate- or arachidonic acid-induced platelet aggregation. After 4 weeks of treatment with the flavonoid extract at a dose of 450 mg/day, a significant reduction in platelet aggregation was observed compared with placebo or baseline values. However, in our study using Pycnogenol

200 mg q.d., we did not find any difference in shear stress-dependent platelet function, which suggests that higher dosages of Pycnogenol are needed to inhibit platelet function.

Recent studies suggested a blood pressure-lowering effect of Pycnogenol. In a double-blind, placebo-controlled cross-over study involving 11 patients, supplementation with Pycnogenol 200 mg q.d. significantly reduced systolic blood pressure of patients with mild-to-moderate hypertension from  $139.9 \pm 3.3$  to  $132.7 \pm 4.18$  mmHg ( $P < 0.05$ ) after 8 weeks of therapy, while diastolic blood pressure remained stable ( $93.8 \pm 1.23$  vs.  $92 \pm 1.7$  mmHg,  $P = \text{NS}$ ).<sup>48</sup> In another double-blind, placebo-controlled trial randomizing 58 hypertensive subjects, intake of 100 mg/day Pycnogenol allowed to reduce the dosage of the calcium channel blocker nifedipine required to maintain normal blood pressure levels.<sup>45</sup> However, we did not find any change in systolic or diastolic blood pressure in our study. This may be explained, at least in part by the fact, that the blood pressure in our study was well controlled at study entry.

## Conclusion

This study demonstrates for the first time an improvement of endothelial function after 8-week treatment with Pycnogenol at a dose of 200 mg q.d. in patients with stable CAD. Pycnogenol significantly reduced oxidative stress as assessed by plasma levels of 8-isoprostanes, but left ADMA and SDMA as well as plasma ET-1 levels unaffected. The clinical implications of these antioxidative properties of the flavonoid extract need to be confirmed in large-scale clinical outcome trials.

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**Conflict of interest:** none declared.

## References

1. Maritime Pine Extract. *USP 34-NF 29 The United States Pharmacopeia and National Formulary*. Maryland: The United States Pharmacopeial Convention Inc.; 2011. p1196–1197.
2. Rohdewald P. Pycnogenol. In: Rice-Evans C, Packer L, eds. *Flavonoids in Health and Disease*. New York: Marcel Dekker Ltd; 1998. p405–419.
3. Grimm T, Schäfer A, Högger P. Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). *Free Radic Biol Med* 2004;**36**:811–822.
4. Das NP. Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochem Pharmacol* 1971;**20**:3435–3445.
5. Sanchez-Patan F, Chioua M, Garrido I, Cueva C, Samadi A, Marco-Contelles J, Moreno-Arribas MV, Bartolome B, Monagas M. Synthesis, analytical features, and biological relevance of 5-(3',4'-dihydroxyphenyl)-gamma-valerolactone, a microbial metabolite derived from the catabolism of dietary flavan-3-ols. *J Agric Food Chem* 2011;**59**:7083–7091.
6. Düweler KG, Rohdewald P. Urinary metabolites of French maritime pine bark extract in humans. *Pharmazie* 2000;**55**:364–368.
7. Grimm T, Skrabala R, Chovanova Z, Muchova J, Sumegova K, Liptakova A, Durackova Z, Hogger P. Single and multiple dose pharmacokinetics of maritime pine bark extract (Pycnogenol) after oral administration to healthy volunteers. *BMC Clin Pharmacol* 2006;**6**:4.
8. Grimm T, Chovanova Z, Muchova J, Sumegova K, Liptakova A, Durackova Z, Högger P. Inhibition of NF-kappaB activation and MMP-9 secretion by plasma of human volunteers after ingestion of maritime pine bark extract (Pycnogenol). *J Inflamm (Lond)* 2006;**3**:1.
9. Schäfer A, Chovanova Z, Muchova J, Sumegova K, Liptakova A, Durackova Z, Högger P. Inhibition of COX-1 and COX-2 activity by plasma of human volunteers after ingestion of French maritime pine bark extract (Pycnogenol). *Biomed Pharmacother* 2006;**60**:5–9.
10. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radic Biol Med* 1999;**27**:704–724.
11. Fitzpatrick DF, Bing B, Rohdewald P. Endothelium-dependent vascular effects of Pycnogenol. *J Cardiovasc Pharmacol* 1998;**32**:509–515.
12. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999;**130**:461–470.
13. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002;**39**:257–265.
14. Deanfield J, Donald A, Ferri C, Giannattasio C, Halcox J, Halligan S, Lerman A, Mancía G, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Schiffrin EL, Taddei S, Webb DJ. Endothelial function and dysfunction. Part I: Methodological issues for assessment in the different vascular beds: a statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension. *J Hypertens* 2005;**23**:7–17.
15. Sudano I, Flammer AJ, Periat D, Enseleit F, Hermann M, Wolfmum M, Hirt A, Kaiser P, Hurlimann D, Neidhart M, Gay S, Holzmeister J, Nussberger J, Mocharfa P, Landmesser U, Haile SR, Corti R, Vanhoutte PM, Luscher TF, Noll G, Ruschitzka F. Acetaminophen increases blood pressure in patients with coronary artery disease. *Circulation* 2010;**122**:1789–1796.
16. Faïta F, Masi S, Loukogeorgakis S, Gemignani V, Okorie M, Bianchini E, Charakida M, Demi M, Ghiadoni L, Deanfield JE. Comparison of two automatic methods for the assessment of brachial artery flow-mediated dilation. *J Hypertens* 2011;**29**:85–90.
17. Gemignani V, Bianchini E, Faïta F, Giannarelli C, Plantinga Y, Ghiadoni L, Demi M. Ultrasound measurement of the brachial artery flow-mediated dilation without ECG gating. *Ultrasound Med Biol* 2008;**34**:385–391.
18. Gemignani V, Faïta F, Ghiadoni L, Poggiani E, Demi M. A system for real-time measurement of the brachial artery diameter in B-mode ultrasound images. *IEEE Trans Med Imaging* 2007;**26**:393–404.
19. Wood WG, Ludemann J, Mitusch R, Heinrich J, Maass R, Frick U. Evaluation of a sensitive immunoluminometric assay for the determination of C-reactive protein (CRP) in serum and plasma and the establishment of reference ranges for different groups of subjects. *Clin Lab* 2000;**46**:131–140.
20. Schulze F, Wesemann R, Schwedhelm E, Sydow K, Albsmeier J, Cooke JP, Boger RH. Determination of asymmetric dimethylarginine (ADMA) using a novel ELISA assay. *Clin Chem Lab Med* 2004;**42**:1377–1383.
21. Mancía G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, Grassi G, Heagerty AM, Kjeldsen SE, Laurent S, Narkiewicz K, Ruilope L, Rynkiewicz A, Schmieder RE, Boudier HA, Zanchetti A, Vahanian A, Camm J, De Caterina R, Dean V, Dickstein K, Filippatos G, Funck-Brentano C, Hellemans I, Kristensen SD, McGregor K, Sechtem U, Silber S, Tendera M, Widimsky P, Zamorano JL, Erdine S, Kiowski W, Agabiti-Rosei E, Ambrosioni E, Lindholm LH, Viigimaa M, Adamopoulos S, Agabiti-Rosei E, Ambrosioni E, Bertomeu V, Clement D, Erdine S, Farsang C, Gaita D, Lip G, Mallion JM, Manolis AJ, Nilsson PM, O'Brien E, Ponikowski P, Redon J, Ruschitzka F, Tamargo J, van Zwieten P, Waeber B, Williams B. 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007;**25**:1105–1187.
22. Flammer AJ, Hermann F, Sudano I, Spieker L, Hermann M, Cooper KA, Serafini M, Luscher TF, Ruschitzka F, Noll G, Corti R. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation* 2007;**116**:2376–2382.
23. Chenevard R, Hurlimann D, Bechir M, Enseleit F, Spieker L, Hermann M, Riesen W, Gay S, Gay RE, Neidhart M, Michel B, Luscher TF, Noll G, Ruschitzka F. Selective COX-2 inhibition improves endothelial function in coronary artery disease. *Circulation* 2003;**107**:405–409.
24. Senn S. *Cross-over Trials in Clinical Research*. Chichester: John Wiley; 1993.
25. Hills M, Armitage P. The two-period cross-over clinical trial. *Br J Clin Pharmacol* 1979;**8**:7–20.
26. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Statist* 1979;**6**:65–70.
27. Kanai H, Hirakata H, Nakayama M, Nagashima A, Fujishima M. Minimal daily variations of plasma and urinary endothelin-1 in healthy subjects. *Clin Nephrol* 1996;**46**:353–354.
28. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation* 2007;**115**:1285–1295.

29. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *Int J Cardiovasc Imaging* 2010;**26**:631–640.
30. Nishioka K, Hidaka T, Nakamura S, Umemura T, Jitsuiki D, Soga J, Goto C, Chayama K, Yoshizumi M, Higashi Y. Pycnogenol, French maritime pine bark extract, augments endothelium-dependent vasodilation in humans. *Hypertens Res* 2007;**30**:775–780.
31. Munzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J* 2010;**31**:2741–2748.
32. Patrignani P, Tacconelli S. Isoprostanes and other markers of peroxidation in atherosclerosis. *Biomarkers* 2005;**10**(Suppl. 1):S24–S29.
33. Wassmann S, Wassmann K, Nickenig G. Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension* 2004;**44**:381–386.
34. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 2003;**108**:1912–1916.
35. Roberts LJ 2nd, Morrow JD. Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cell Mol Life Sci* 2002;**59**:808–820.
36. Ryan J, Croft K, Mori T, Wesnes K, Spong J, Downey L, Kure C, Lloyd J, Stough C. An examination of the effects of the antioxidant Pycnogenol on cognitive performance, serum lipid profile, endocrinological and oxidative stress biomarkers in an elderly population. *J Psychopharmacol* 2008;**22**:553–562.
37. Noda Y, Anzai K, Mori A, Kohno M, Shinmei M, Packer L. Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR30 ESR spectrometer system. *Biochem Mol Biol Int* 1997;**42**:35–44.
38. Bayeta E, Lau BHS. Pycnogenol inhibits generation of inflammatory mediators in macrophages. *Nutr Res* 2000;**20**:249–259.
39. Devaraj S, Vega-Lopez S, Kaul N, Schonlau F, Rohdewald P, Jialal I. Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. *Lipids* 2002;**37**:931–934.
40. Anderssohn M, Schwedhelm E, Luneburg N, Vasan RS, Boger RH. Asymmetric dimethylarginine as a mediator of vascular dysfunction and a marker of cardiovascular disease and mortality: an intriguing interaction with diabetes mellitus. *Diab Vasc Dis Res* 2010;**7**:105–118.
41. Boger RH, Maas R, Schulze F, Schwedhelm E. Elevated levels of asymmetric dimethylarginine (ADMA) as a marker of cardiovascular disease and mortality. *Clin Chem Lab Med* 2005;**43**:1124–1129.
42. Mangoni AA. The emerging role of symmetric dimethylarginine in vascular disease. *Adv Clin Chem* 2009;**48**:73–94.
43. Schulze F, Carter AM, Schwedhelm E, Ajjan R, Maas R, von Holtzen RA, Atzler D, Grant PJ, Boger RH. Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke. *Atherosclerosis* 2010;**208**:518–523.
44. Spieker LE, Flammer AJ, Luscher TF. The vascular endothelium in hypertension. *Handb Exp Pharmacol* 2006;**176**:249–283.
45. Liu X, Wei J, Tan F, Zhou S, Wurthwein G, Rohdewald P. Pycnogenol, French maritime pine bark extract, improves endothelial function of hypertensive patients. *Life Sci* 2004;**74**:855–862.
46. Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. *Int J Clin Pharmacol Ther* 2002;**40**:158–168.
47. Peng Q, Wei Z, Lau BH. Pycnogenol inhibits tumor necrosis factor- $\alpha$ -induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. *Cell Mol Life Sci* 2000;**57**:834–841.
48. Hosseini S, Jeongmin L, Sepulveda RT, Rohdewald P, Watson RR. A randomized, double-blind, placebo-controlled, prospective, 16 week crossover study to determine the role of Pycnogenol in modifying blood pressure in mildly hypertensive patients. *Nutr Res* 2001;**21**:1251–1260.
49. Lau BH, Riesen SK, Truong KP, Lau EVV, Rohdewald P, Barreta RA. Pycnogenol as an adjunct in the management of childhood asthma. *J Asthma* 2004;**41**:825–832.
50. Araghi-Niknam M, Hosseini S, Larson D, Rohdewald P, Watson RR. Pine bark extract reduces platelet aggregation. *Integr Med* 2000;**2**:73–77.